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Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia

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An *ex vivo* approach to gene therapy for familial hypercholesterolaemia (FH) has been developed in which the recipient is transplanted with autologous hepatocytes that are genetically corrected with recombinant retroviruses carrying the LDL receptor. We describe the treatment of a 29 year old woman with homozygous FH by *ex vivo* gene therapy directed to liver. She tolerated the procedures well and *in situ* hybridization of liver tissue four months after therapy revealed evidence for engraftment of transgene expressing cells. The patient's LDL/HDL ratio declined from 10–13 before gene therapy to 5–8 following gene therapy, improvements which have remained stable for the duration of the treatment (18 months). This represents the first report of human gene therapy in which stable correction of a therapeutic endpoint has been achieved.

Familial hypercholesterolaemia (FH) has emerged as an important model for the development of human gene therapies[1]. This disorder, caused by inherited deficiency of LDL receptors, is associated with severe hypercholesterolaemia and premature coronary artery disease[1]. The homozygous form of FH is an excellent candidate for early applications of gene therapy because it is a lethal disorder that is refractory to conventional therapies. Measurement of serum lipid profiles provides a convenient and clinically relevant endpoint to evaluate response to therapy, and orthotopic liver transplantation has been shown to correct the underlying dyslipidemia indicating that hepatic reconstitution of LDL receptor expression is sufficient for metabolic correction[2,3].

The original paradigm for liver-directed gene therapy was based on transplantation of autologous hepatocytes genetically modified *ex vivo* with recombinant retroviruses. The efficacy and safety of this approach for treatment of FH has been demonstrated in a variety of animal models. A strain of rabbits genetically deficient in LDL receptors, called the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, was used to demonstrate the potential efficacy of *ex vivo* gene therapy. Analysis of recipient animals demonstrated stable engraftment of genetically modified hepatocytes and persistent reductions in serum cholesterol for the duration of the experiment — 6.5 months[4]. Experiments in larger animals including dogs and baboons documented the feasibility and safety of *ex vivo* gene therapy directed to the liver[5,6]. *In situ* hybridization analysis of liver tissue from baboons harvested 1.5 years after gene therapy demonstrated stable engraftment of transgene expressing hepatocytes, providing further support for the efficacy of this therapy (unpublished data).

Based on the encouraging results obtained in animal models, we proposed a clinical protocol to treat FH homozygous patients with *ex vivo* gene therapy. We received permission from the US Recombinant DNA Advisory Committee (RAC) and the US Food and Drug Administration to treat three patients who had developed overt coronary artery disease and therefore would have a poor prognosis. Our experience with the first patient, described here, supports the efficacy and safety of liver-directed *ex vivo* gene therapy in humans.

First recipient of liver-directed gene therapy

The first clinical application of liver-directed gene therapy in humans used the *ex vivo* approach in a patient with homozygous FH. Although we were allowed to treat FH patients of any age, the RAC suggested that we enroll an adult as the initial patient to simplify the informed consent process. Patient FH1 underwent gene therapy on June 5, 1992. This French Canadian woman, who at the time of gene therapy was 28 years old, had a myocardial infarction at the age of 16 and required coronary artery bypass at the age of 26. Her dyslipidemia — which at baseline included a total serum cholesterol concentration of 545 mg dl^{-1}, LDL of 482 mg dl^{-1} and HDL of 43 mg dl^{-1} — was refractory to treatment with a variety of drugs including HMG CoA reductase inhibitors and bile acid binding resins. Genotype analysis indicated she was homozygous for a missense mutation (Trp66Gly, exon 3) that renders the LDL receptor incapable of binding to its ligands[7]. Cardiac evaluation performed prior to gene therapy revealed failure of one of her grafts and diffuse disease in her native coronary arteries, however, she was not overtly symptomatic.

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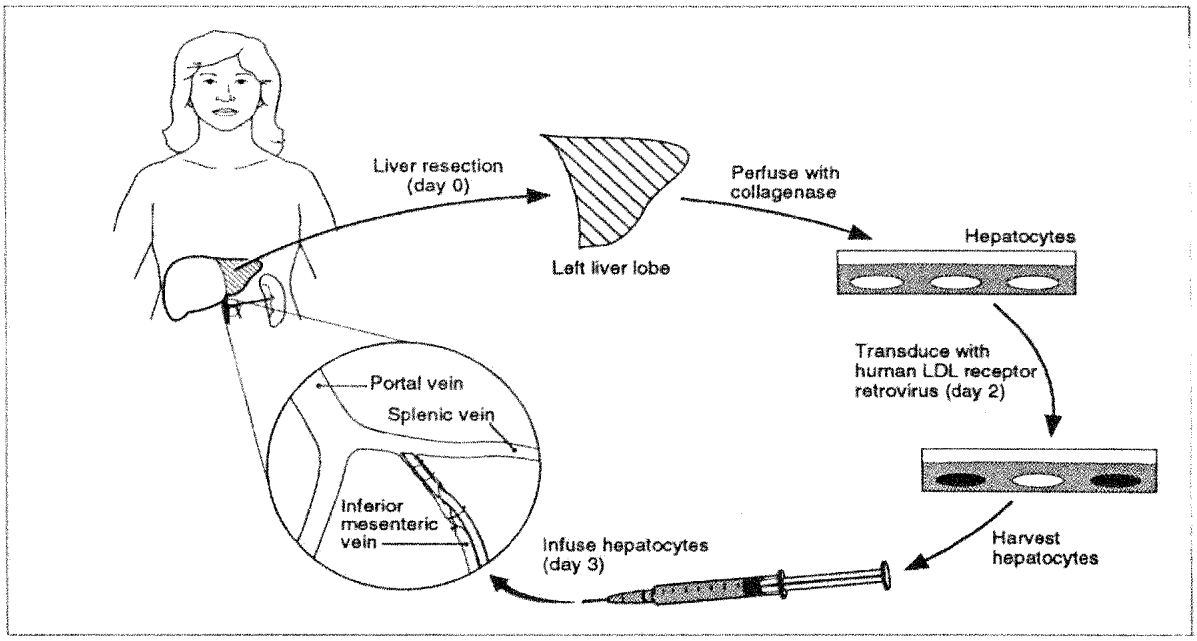

Fig. 1 Strategy of *ex vivo* gene therapy for familial hypercholesterolaemia.

Ex vivo gene therapy to liver is feasible and safe

The clinical protocol approved by the RAC has been published[8]; the general strategy is summarized in Fig. 1. The left lateral segment of the patient's liver, comprising approximately 15% of its total mass, was removed through a left subcostal incision. A 9.6 fr Hickman catheter was inserted into her inferior mesenteric vein, and the distal end of the catheter was brought through her incision thereby providing convenient access to the portal circulation for subsequent cell infusions. The resected liver, weighing 250 g, was perfused with collagenase to release hepatocytes; 3.2×10^9 cells were recovered (98% viability) and seeded into 800 10 cm^2 plates. Medium containing the LDL receptor expressing recombinant retroviruses was placed onto the cultured hepatocytes 48 hours after the initial seeding. Following a 12–18 h exposure to virus, the cells were analysed for LDL receptor expression and harvested for transplantation; 2×10^9 viable cells were recovered from the plates by treatment with trypsin. Incubation of the transduced cells with fluorescent labelled LDL revealed uptake in approximately 20% of the cells exposed to the LDL receptor expressing virus and no uptake in duplicate plates of cells not exposed to virus (Fig. 2).

Prior to infusion of the cells, a portal venogram was performed to confirm the placement of the catheter and patency of the portal circulation (Fig. 3*a*). The genetically corrected hepatocytes were harvested in three aliquots and each aliquot was manually infused at 4 h intervals directly into the catheter over a 30 minute period (a rate of ~2 cc min^{-1}). During the cell infusions the patient was carefully monitored in the intensive care unit; her vital signs measured during this time are presented in Fig. 4. She tolerated the cell infusions well except for a transient tachycardia early in the day, thought to be secondary to anxiety, and fevers that were present before cell infusion which resolved subsequently.

One concern was the potential development of portal

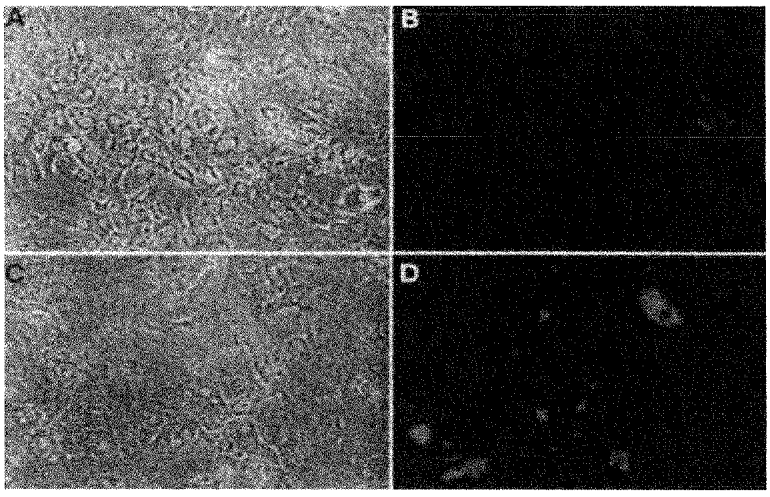

Fig. 2 Expression of recombinant LDL receptor in primary hepatocytes. Primary cultures of hepatocytes were infected with recombinant retroviruses and analysed for LDL receptor expression using an assay based on uptake of fluorescent labelled LDL. Mock infected cells visualized under the phase contrast (*a*) and fluorescent microscope (*b*), and LDL receptor transduced cells visualized under the phase contrast (*c*) and fluorescent microscope (*d*) are presented. Magnification, 100×.

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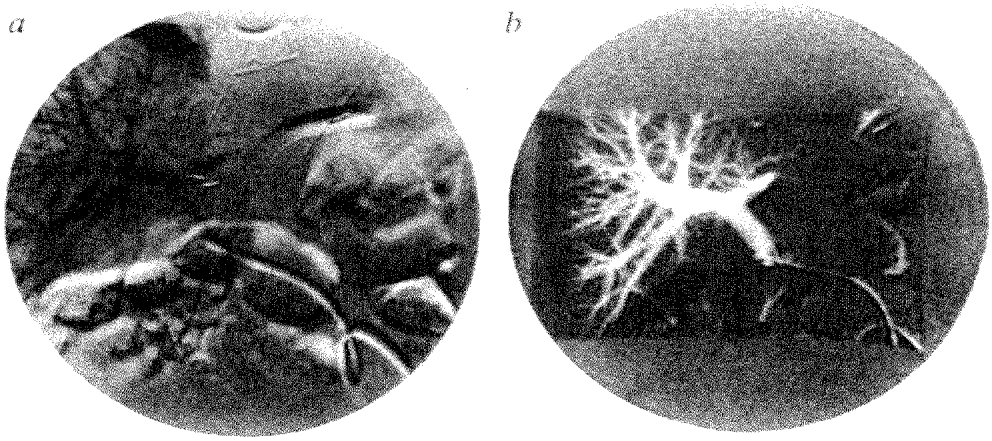

Fig. 3 Portal venograms before and after hepatocyte infusion. *a*, Portal venogram immediately prior to cell reinfusion (postoperative day 3). Note surgical absence of the left lateral segment of the liver, absence of portal vein thrombosis and good position of the catheter. *b*, Portal venogram immediately prior to catheter removal (postoperative day 10). Note patent portal vein without evidence of intraluminal thrombus.

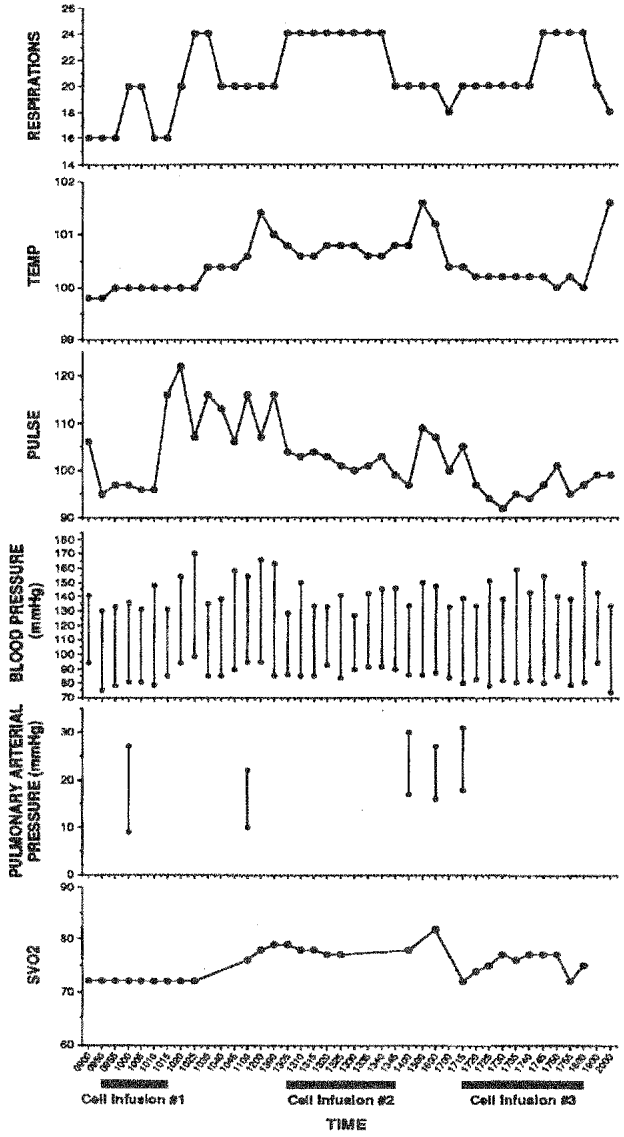

Fig. 4 Clinical response during hepatocyte infusion. During the cell infusions the patient was invasively monitored with a radial arterial line and pulmonary arterial line while in the intensive care unit. Six clinical parameters (respiration rate, oral temperature, pulse, systemic blood pressure (mm Hg), pulmonary arterial pressure (mm Hg), and oxygen saturation in mixed venous blood (SV02)) are presented. The actual times are indicated along the bottom with the periods during which the cells were infused indicated by the bars. Note that the time coordinates have been expanded during the periods of cell infusion.

vein thrombosis and/or portal hypertension as a result of introducing a large cell mass into this low pressure venous circulation. Portal pressures measured via the catheter three days before cell infusion (9.8 ± 1.3, mean ± s.d., n=4) were indistinguishable from those measured five days after cell infusion (10.9 ± 2.1 mean ± s.d. n=12) with transient increases (lasting <4 h) of 4 and 8 cm H_20 occurring after the second and third cell infusions, respectively. Repeat portal venography performed at the time of catheter removal seven days after cell infusion revealed a fully patent portal circulation without evidence of intraluminal clot (Fig. 3*b*).

Prolonged improvement in dyslipidemia

Liver tissue was harvested by percutaneous biopsy four months after gene therapy. No histopathology was noted in plastic embedded sections prepared for light and electron microscopy (data not shown). Frozen sections were analysed for the presence of transgene expressing cells by *in situ* hybridization using an RNA probe specific for the recombinant derived LDL receptor transcript. Figure 5 presents an example of an hepatocyte that hybridized to the antisense probe (*c* and *d*); this kind of focal hybridization was not present in serial sections incubated with the sense probe (*a* and *b*) or in sections pretreated with RNase prior to hybridization with the antisense probe (data not shown). Analysis of a limited number of sections revealed hybridization to the antisense probe in 1:1,000 to 1:10,000 cells. It is unlikely that the results obtained from a single small block of liver tissue from FH1 accurately represents the abundance and distribution of transgene expressing cells throughout the liver. Similar experiments performed in three baboons who underwent

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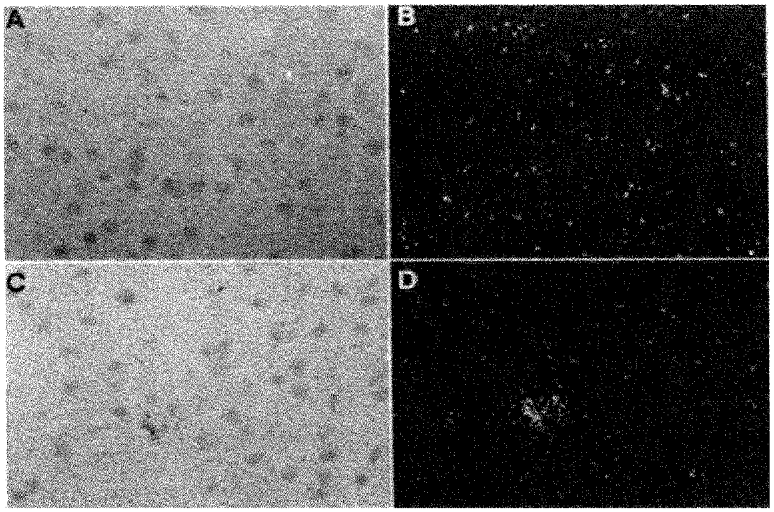

Fig. 5 *In situ* hybridization of liver tissue after gene therapy. Liver tissue (50 mg) was harvested by percutaneous biopsy four months after gene therapy. The majority of the sample was fixed, embedded, sectioned and analysed by light and electron microscopy for evidence of pathology. A small block was analysed for cells expressing recombinant LDL receptor by *in situ* hybridization. Tissue sections were hybridized with the sense probe (*a* and *b*) or antisense probe (*c* and *d*) and visualized by bright field (left panels) and dark field (right panels) microscopy. The clustering of signal seen in panels *c* and *d* indicates a cell that hybridized to the antisense probe. Magnification, 50×.

ex vivo liver-directed gene therapy demonstrated marked regional variation of recombinant LDL receptor expressing cells within large biopsies of liver, illustrating the limitations in quantitatively assessing engraftment from the small quantity of tissue sampled from a percutaneous biopsy (unpublished data).

The effect of gene therapy on the patient's lipid profiles, presented as ΔLDL, ΔHDL and LDL/HDL ratio, is presented in Fig. 6; Table 1 summarizes the lipid data with relevant statistical analyses. The patient has been followed for 18 months after gene therapy in the context of four treatment periods: pre gene therapy — on (period A) and off (period B) lovastatin; and post gene therapy — off (period C) and on (period D) lovastatin. The original protocol was designed to establish baseline lipids when the patient was off all lipid lowering medications and to reinitiate pharmacologic therapy four months after gene therapy. This initial analysis allowed comparisons between periods B, C and D.

Blood samples were coded and submitted to a reference

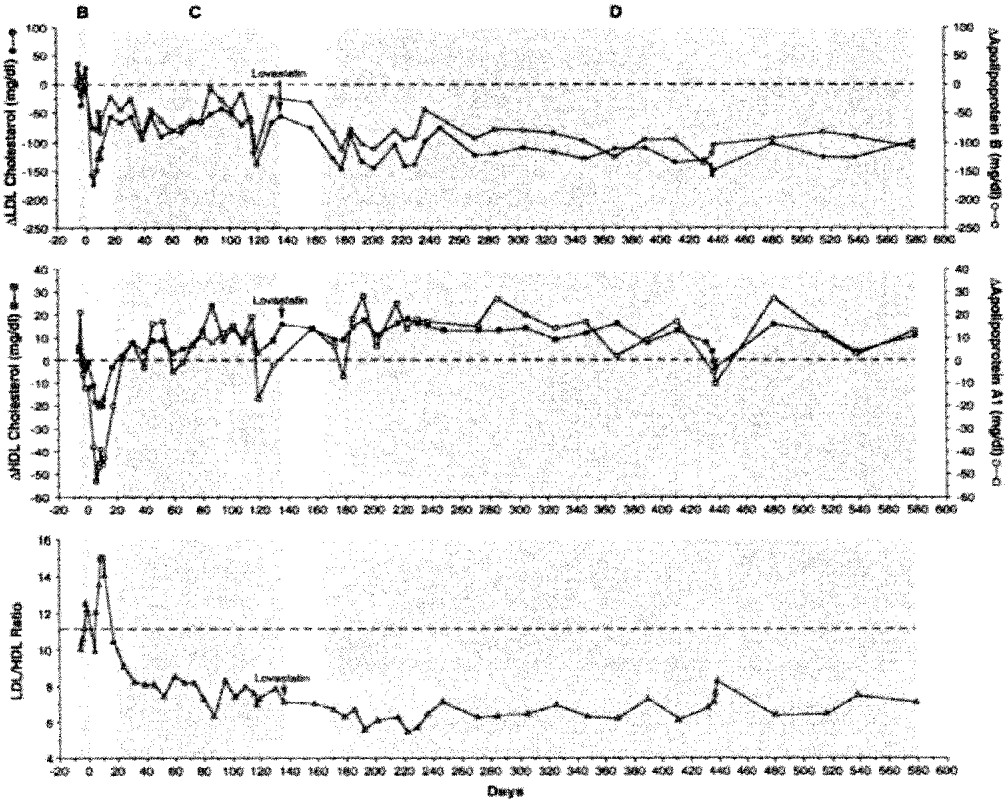

Fig. 6 Lipid profiles. The study was performed using three treatment periods. Period B spans 8 days immediately prior to gene therapy during which 7 lipid profiles were obtained. Period C represents a 131 day interval after gene therapy before she was started on lovastatin during which 19 lipid profiles were obtained. Interpretation of data obtained 8 days following gene therapy was confounded because of additional effects on lipids of the stress of the procedure and decreased nutritional intake; these data were deleted from the analysis of Period C. Period D represents a 15 month interval following Period C during which the patient was treated with lovastatin. Data obtained during a 30 day period after initiation of lovastatin was not included in analysis of Period D to allow for the effect of the drug. Data are presented as ΔLDL and Δapo B (top panel); ΔHDL and Δapo AI (middle panel); and LDL/HDL ratio (bottom panel). Three treatment periods are indicated: period B — pre-gene therapy, off medications; period C — post-gene therapy, off medications; and period D — post-gene therapy, on lovastatin.

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Table 1 Summary of lipid profiles for patient FH1

	Pre-gene therapy		Post-gene therapy		Statistical comparisons (p values)		
	+Lovastatin period A	–Lovastatin period B	–Lovastatin period C	+Lovastatin period D	B–C	C–D	A–D
Cincinnati ref. lab.							
LDL	–	482 ± 19 (7)	404 ± 24 (19)	356 ± 22 (27)	0.0001	0.0001	–
HDL	–	43 ± 3.4 (7)	51.4 ± 5.9 (19)	54 ± 5 (27)	0.0014	0.10	–
LDL/HDL	–	11 ± 1.0 (7)	7.9 ± 0.9 (19)	6.6 ± 0.6 (27)	0.0001	0.0001	–
apoAI	–	115 ± 12 (7)	121 ± 11 (18)	130 ± 9 (24)	0.22	0.005	–
apoB	–	352 ± 23 (7)	299 ± 30 (18)	260 ± 23 (24)	0.0004	0.0002	–
Quebec ref. lab.							
LDL	448 ± 30 (6)	–	–	366 ± 25 (7)	–	–	0.0001
HDL	44.5 ± 4.1 (6)	–	–	47.6 ± 4.1 (7)	–	–	0.16
LDL/HDL	10.2 ± 1.0 (6)	–	–	7.6 ± 0.6 (7)	–	–	0.0001

All samples represent mean ± 1. s.d. with *n*=number of determinations. Statistical analyses performed as described in the text.

laboratory in Cincinnati for analysis. Serum LDL dropped by 180 mg dl^{-1} immediately after cell infusion and regained a new baseline that was 17% lower than pre treatment levels (482±19 before therapy versus 404 ± 24 after therapy, p=0.0001). Coincident with the diminution in LDL was an increase in HDL from 43 ± 3.4 to 51.4 ± 5.9 (p=0.0014) that translated to a decline in LDL/HDL ratio from 11 ± 0.4 to 7.9 ± 0.9 (p=0.0001). The mechanism(s) responsible for increased HDL following gene therapy remain unexplained, however, similar effects have been described in FH homozygotes who underwent orthotopic liver transplantation[2,3]. Initiation of lovastatin four months after gene therapy was associated with further improvements in this patient's dyslipidemia including a reduction in LDL (404 ± 24 to 356 ± 22, p=0.0001), increase in HDL (51.4 ± 5.9 to 54 ± 5, p=0.10), and decline in LDL/HDL ratio (7.9 ± 0.9 to 6.6 ± 0.6, p=0.0001). The changes in LDL and HDL noted in each treatment period were associated with parallel and equally significant changes in apo B and apo AI, respectively (Table 1 and Fig. 6).

An attempt was made to evaluate further the effect of gene therapy using lipid profiles that were obtained in Quebec during period A, spanning a two year interval from Feb. 1990–Dec. 1991; during this time the patient was treated with lovastatin in a single drug regimen at approximately the same dose she has been taking during period D (that is, following gene therapy). Additional samples were obtained during period D and analysed by the same reference laboratory in Quebec that was used to measure the patient's lipids in 1990–91. Direct comparison of the patient's lipids on lovastatin before and after gene therapy revealed a diminution in LDL from 448 ± 30 to 366 ± 25 (p=0.0001), a modest increase in HDL from 44.5 ± 4.1 to 47.6 ± 4.1 (p=0.16), and a decline in LDL/HDL ratio from 10.2 ± 1.0 to 7.6 ± 0.6 (p=0.0001).

Discussion

FH represents a unique model for developing and evaluating the principle of liver-directed gene therapy in humans. This fatal disease is easily evaluated for reconstitution of hepatic gene expression by serial measurements of serum lipids which are considered relevant endpoints for clinical efficacy. Critical to the early development of this clinical model was the availability of an authentic animal model, the WHHL rabbit.

Ex vivo approaches to liver-directed gene therapy emerged as the initial paradigm for treating hepatic metabolic diseases such as FH. In this strategy, stable reconstitution of hepatic gene expression can be achieved by transplanting hepatocytes transduced *ex vivo* with retroviruses. The development of safe and effective *ex vivo* gene therapies to liver presents unique experimental challenges. *Ex vivo* correction of the defect is complicated because the target cell for gene transfer, the hepatocyte, must be isolated from surgically resected tissue and it cannot be maintained and expanded in culture. The ultimate success of this approach depends on the efficient and stable engraftment of the transduced cells and their progeny. The likelihood that this will occur with transduced hepatocytes is difficult to predict because of the paucity of information available regarding stem cells and lineage in the liver, and ultimately must be answered experimentally. Clinical application of this form of gene therapy was further confounded because it does not resemble existing forms of therapy as is the case with bone marrow directed gene therapy, which conceptually is a modification of a commonly used therapy, autologous bone marrow transplantation. However, there should be no immunological barriers associated with *ex vivo* gene therapy other than the problem of an immune response to the therapeutic gene product, a potential concern that is generic to all forms of gene therapy for deficiency states. A variety of animal models, in addition to the WHHL rabbit, have been useful in developing the requisite technology and providing sufficient preclinical studies to justify a human trial[4,9,10].

The outcome of our first clinical experience supports the safety and feasibility of *ex vivo* gene therapy directed to liver. Molecular and metabolic data suggest that the genetically modified hepatocytes have engrafted stably in this patient and continue to express the recombinant gene (after at least 18 months). The level of metabolic correction achieved in this patient was similar to that detected in the WHHL rabbits who received autologous genetically corrected hepatocytes[4]. In this animal model, control experiments performed with mock transfected hepatocytes had no effect on cholesterol except for a transient elevation suggesting that the persistent diminution in lipoproteins observed in FH1 was not an artefact of the surgical

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procedures but due to expression of the recombinant gene. Subsequent to gene therapy, the patient's serum lipids consistently remained at levels significantly lower than those measured by at least two reference laboratories over several years before gene therapy. It is unclear, however, whether the partial correction of hypercholesterolaemia achieved in this patient will translate to improved clinical outcome. It is encouraging that she tolerated gene therapy well without short or long term sequelae and that her coronary artery disease, as documented by serial angiography, has not progressed during the 18 months since the treatment (data not shown).

The response of this patient to lovastatin following gene therapy is interesting given that she failed to respond to this drug on multiple occasions prior to gene therapy. Lovastatin is thought to deplete intracellular cholesterol which leads to up regulation of LDL receptor expression[1], probably at the transcriptional level[11]. The recombinant LDL receptor gene does not contain the transcriptional elements necessary to confer cholesterol mediated regulation suggesting the response to lovastatin is unrelated to the recombinant gene or that its effect is in part mediated by posttranscriptional regulation of LDL receptor. This is consistent with previous studies that indicate the endogenous LDL receptor gene is regulated at both a transcriptional and posttranscriptional manner[12].

One potential concern about gene therapy for diseases caused by loss of gene function is that the protein product of the therapeutic gene will be recognized by the recipient as a neoantigen leading to an immune response against the genetically corrected cells. Several observations suggest this did not occur in FH1. Western blot analysis of the patient's sera failed to detect antibodies to the recombinant human LDL receptor protein (data not shown). Also, there was no clinical or pathological evidence for autoimmune hepatitis following gene therapy. It will be interesting to see if similar results are obtained in FH patients undergoing gene therapy who have mutations that totally ablate LDL receptor protein expression as opposed to the mutation in FH1 that leads to the expression of a dysfunctional protein[7].

Our study demonstrates the feasibility, safety and potential efficacy of *ex vivo* liver-directed gene therapy in humans and supports the initial hypothesis that selective reconstitution of LDL receptor expression in hepatocytes of FH homozygotes should be sufficient for metabolic improvement. This represents the first example of stable correction of a therapeutic endpoint by gene therapy, in contrast to clinical trials that require repeated administration of short-lived target cells such as lymphocytes for treatment of adenosine deaminase deficiency. Translation of this technology to the treatment of other lethal liver metabolic diseases (such as, ornithine transcarbamylase deficiency) should proceed rapidly if the principle of *ex vivo* liver-directed gene therapy is confirmed in a larger number of homozygous FH patients. Ultimately, a more effective and clinically practical approach to liver directed gene therapy, based on *in vivo* gene delivery, must be developed. Gene transfer technologies using recombinant adenoviruses, liposomes and molecular conjugates have shown promising results in animal models[13–15]. Problems with efficiency and stability of recombinant gene expression as well as destructive and/or blocking immune responses to the delivery vehicles or genetically modified cells must be overcome before the potential of *in vivo* approaches can be realized.

Methodology

Surgical procedures. During the procedure and for the first three postoperative days the patient was carefully monitored with a pulmonary arterial catheter and radial arterial line. Following induction of anesthaesia, the left lobe of the liver was exposed by a left subcostal incision which was extended up the midline to the xiphoid. A self-retaining retractor was used to retract the costal margin. The left triangular ligament was divided to the level of the left hepatic vein from lateral to medial. A rubber-shod, non-crushing intestinal bowel clamp was tested for fit just to the left of the falciform ligament. The clamp was applied and using a scalpel, the liver surface was rapidly transected (<90 s) and transferred to the human applications laboratory for cell isolation. Bleeding from the cut hepatic vein was easily controlled with direct pressure until surgical hemostasis was applied. The cut ends of the portal vein and hepatic vein were sutured with 5-0 running non-absorbable monofilament. The open surface of the liver was controlled with 3-0 silk interlocking vertical mattress sutures placed in the liver tissue protruding from the clamp. Once haemostasis was achieved, the clamp was removed and individual bile ducts or blood vessels were ligated with additional 3-0 silk sutures. The inferior mesenteric vein was identified at the paraduodenal fossa. The vein was sharply dissected for a distance of 3 cm and individual branches were ligated with 5-0 silk ligatures. Silk ties (2-0) were placed at either end of the dissected vessel, and the ligature placed at the end of the vein nearest to the colon was tied. A 9.6 Fr. Hickman-type catheter was brought obliquely through the abdominal wall about 3 cm below the lateral aspect of the incision and secured in place with a 3-0 nylon stitch. The catheter was trimmed to the correct length, a bevel was placed at the cut end to facilitate insertion, and the catheter was primed with heparinized saline (100 U ml^{-1}). A venotomy was made with a number 11 scalpel blade. The ideal location for catheter placement is the confluence of the inferior mesenteric vein and the splenic vein, a position that was identified by palpation. The catheter was secured by tying the ligature nearest the portal vein around the inferior mesenteric vein making sure not to occlude the catheter. A 3-0 chromic suture was tied around the inferior mesenteric vein and the outside of the catheter to further protect against premature dislodgement (see Fig. 1). A final inspection of the cut surface of the liver was made and the liver bed was drained with a closed suction drainage catheter to remove any residual bile or serum. The wound was closed in two fascial layers with a running absorbable monofilament suture. The skin was closed with interrupted subcuticular 4-0 chromic sutures and surgical tapes.

Preparation of virus and isolation of hepatocytes. The recombinant retrovirus used in this study has been described[6]. A full length human LDL receptor cDNA is expressed from a chicken β-actin promoter in combination with an enhancer from the immediate early gene of cytomegalovirus. The cell line that produced this virus, called 132-10, was characterized in accordance with recommendations of the RAC and the FDA. Supernatants containing the LDL receptor viruses were cryopreserved and aliquots were analysed for the presence of contaminants and replication competent virus. Certified lots of cryopreserved virus were used in the clinical trial.

Hepatocytes were isolated by collagenase perfusion, plated in culture and infected with retroviruses as described previously[16]. Plates of cells were infected with virus from 132-10 (LDL receptor virus) and analysed for LDL receptor expression using the previously described assay; cells were incubated in RPMI 1640 medium containing lipoprotein deficient serum (10%) and fluorescent labelled LDL (10 μg ml^{-1}) for 4 h[16]. Following completion of this incubation, the medium was removed, and the cells were washed in PBS and visualized under the fluorescent microscope. In preparation for transplantation, hepatocytes were removed from the plates by incubation with trypsin and washed extensively in RPMI 1640. Hepatocytes were harvested in three batches each of which contained cells recovered from one third of the total prep. Each batch was washed and resuspended in normal saline (50 ml) containing 10 U ml^{-1} of heparin in preparation for infusion.

Analyses of biopsied liver tissue. The tissue block for *in situ* analysis

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was frozen in OCT, and cryosections (6 μM) were mounted on gelatin poly(L-lysine)-coated sides and fixed with 4% paraformaldehyde in phosphate buffered saline[17]. Sections were hybridized to a ^{35}S labelled RNA probe complementary to retroviral envelope sequences that are uniquely present in the 3' untranslated region of the recombinant derived LDL receptor RNA[4]. Sense probes and RNAse pretreatment with antisense probes were used as controls for hybridization specificity.

Analysis of metabolic parameters. Blood samples were obtained, coded and sent to reference laboratories in Cincinnati and Quebec for determination of lipid profiles. LDL cholesterol, HDL cholesterol, ApoA 1 and ApoB were measured directly using previously published techniques. Differences in LDL, HDL and LDL/HDL profiles obtained during the four treatment periods were evaluated using random intervention testing and randomized testing methodologies.

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